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<p>(21) International Application Number: PCT/GB94/01480 (22) International Filing Date: 8 July 1994 (08.07.94) (30) Priority Data: 9314271.9 9 July 1993 (09.07.93) GB (71) Applicants (for all designated States except US): THE INSTITUTE OF CANCER RESEARCH [GB/GB]; 17A Onslow Gardens, London SW7 3AL (GB). THE WELLCOME FOUNDATION LIMITED [GB/GB]; Unicorn House, 160 Euston Road, London NW1 2BP (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): GUSTERSON, Barry, Austin [GB/GB]; Institute of Cancer Research, Royal Cancer Hospital, Haddow Laboratories, 15 Cotswold Road, Sutton, Surrey SM2 5NG (GB). CROMPTON, Mark, Roger [GB/GB]; Institute of Cancer Research, Royal Cancer Hospital, Haddow Laboratories, 15 Cotswold Road, Sutton, Surrey SM2 5NG (GB). MITCHELL, Philip, John [GB/GB]; Institute of Cancer Research, Royal Cancer Hospital, Haddow Laboratories, 15 Cotswold Road, Sutton, Surrey SM2 5NG (GB). BARKER, Karen, Tracey [GB/GB]; Institute of Cancer Research, Royal Cancer Hospital, Haddow Labora-</p>	<p>tories, 15 Cotswold Road, Sutton, Surrey SM2 5NG (GB). MARTINDALE, Jane, Elizabeth [GB/GB]; Institute of Cancer Research, Royal Cancer Hospital, Haddow Laboratories, 15 Cotswold Road, Sutton, Surrey SM2 5NG (GB). PAGE, Martin, John [GB/GB]; The Wellcome Research Laboratories, Langley Court, South Eden Park Road, Beckenham, Kent BR3 3BS (GB). SPENCE, Paul [GB/GB]; The Wellcome Research Laboratories, Langley Court, South Eden Park Road, Beckenham, Kent BR3 3BS (GB). (74) Agent: MARCHANT, James, Ian; Elkington and Fife, Prospect House, 8 Pembroke Road, Sevenoaks, Kent TN13 1XR (GB). (81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: CELL GROWTH FACTOR RECEPTORS (57) Abstract The invention relates to a method of screening a substance for potential utility as a therapeutic agent in the treatment of cancer which comprises providing a standard system in which a protein tyrosine kinase or an active fragment thereof is able to develop a measurable effect, allowing the protein tyrosine kinase to develop that effect in the presence and absence of the said substance and measuring that effect, ability to produce significant inhibition of the effect being taken as an indication of potential utility as a therapeutic agent, wherein the protein tyrosine kinase is characterised by the amino acid sequence of SEQ ID NO. 1 or an amino acid sequence showing a significant degree of homology thereto. The invention also relates to a therapeutic agent thus identified, to a method for detecting the protein tyrosine kinase and an enzyme substrate complex comprising the protein tyrosine kinase and a therapeutic agent capable of modulating the activity of the protein tyrosine kinase.</p>		

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CELL GROWTH FACTOR RECEPTORS

5 The present invention relates to cell growth factor receptors. More particularly the invention relates to the use of a tyrosine kinase growth factor receptor in the development of diagnostic and therapeutic approaches to cancer, for example breast cancer.

10 Protein tyrosine kinases are enzymes which show the property of catalysing the transfer of phosphate groups from donor molecules (ATP) to the hydroxyl groups of tyrosine residues in polypeptides. Known tyrosine kinases can be classified into two broad groups. Transmembrane tyrosine
15 kinases traverse cellular membranes so that they possess extracellular and intracellular domains. Cytoplasmic tyrosine kinases are located only intracellularly. A general feature of transmembrane (also referred to as receptor) tyrosine kinases is that they possess extracellular ligand-binding
20 domains, hydrophobic transmembrane sequences, and intracellular portions which include the tyrosine kinase domains (for a review see Ullrich & Schlessinger Cell, 61, 203-212 (1990)).

25 Tyrosine kinases can induce cell proliferation, cell transformation and regulate developmental events (see reviews by Hanks et al, Science, 241, 42-75 (1988) and Cantley et al, Cell 64, 281-302 (1991)). In general, where functional assays have been available (e.g. mitogenesis or transformation), it
30 has been shown that the biological functions of tyrosine kinases are usually dependent on intact enzyme activity and that through autophosphorylation and the phosphorylation of other proteins, they alter the subcellular localisation and activities of various components of the intracellular
35 signalling pathways.

Analysis of the oncogenes of many acutely transforming animal retroviruses has revealed that their products frequently manifest tyrosine kinase activity, as do the

products of their cellular proto-oncogene counterparts. Other genes encoding tyrosine kinases have been found to be altered by DNA rearrangements in cancer cells, the result being a presumed acquisition of cellular transforming activity (for example c-abl, c-met). Some cellular proto-oncogenes encoding tyrosine kinases have been cloned independently by virtue of the fact that they encode growth factor receptors, for example the epidermal growth factor receptor. On the other hand, the genes for other growth factor receptors which have tyrosine kinase activities, such as those for platelet-derived growth factor and insulin-like growth factors, have been well characterised, but have never been found to be transduced by retroviruses.

The c-erbB-2/HER2/c-neu gene encodes a transmembrane receptor-like tyrosine kinase which is structurally very similar to the EGF/TGF alpha receptor. The c-erbB-2 gene has been found to be overexpressed in 20 to 30% of human breast tumours, often in association with gene amplification, and this phenotype is now generally accepted as predictive of poor disease free and overall survival (for reviews see Sunderland & McGuire in *Regulatory Mechanisms in Breast Cancer*, Lippman & Dickson (Eds.), Kluwer Academic Publishers, Boston, pages 3 to 22 (1991) and Gusterson et al., *J. Clin. Oncol.*, 10, 1049-1056 (1992)). Overexpression of the gene in fibroblasts induces transformation (di Fiore et al, *Science*, 237, 178-182 (1987) and Hudziak et al, *Proc. Natl. Acad. Sci. (USA)*, 84, 7159-7162 (1987)), and ligands which bind to the c-erbB-2 gene product (human and rodent) and activate its tyrosine kinase activity have been identified (Lupu et al, *Science*, 249, 1552-1555 (1990), Dobashi et al, *Proc. Natl. Acad. Sci. (USA)*, 88, 8582-8586 (1991), Wen et al, *Cell*, 69, 559-572 (1992) and Holmes et al, *Science* 256, 1205-1210 (1992)). Some of these ligands increase the rate of proliferation of cells expressing the c-erbB-2 protein. The strong implication of these and other findings is that overexpression of this tyrosine kinase in some breast tumour cells is one of the important steps in their progression towards tumourigenicity, and therefore that c-erbB-2 can function as an oncogene.

A variety of other receptor tyrosine kinases, including those for the IGFs and the FGFs, are expressed in breast tumours (Stewart et al, J. Biol. Chem., 265 21172-21178 (1990) and Wellstein & Lippman in Molecular Foundations of Oncology, Broder (Ed.), Williams and Wilkins, Baltimore, pages 403-418 (1991)), and the EGF/TGF alpha receptor is overexpressed in some cases (for example Horak et al, Oncogene, 6, 2277-2284 (1991)). As with c-erbB-2, overexpression of EGFs has been correlated with poor prognosis. Since the ligands of some of these receptors can be detected in breast tumour samples, it may be that breast tumour development is also regulated by these molecules (for a review see van de Vijver & Nusse, Biochim, Biophys. Acta, 1072, 35-50 (1991)).

The features which are ideally required in a molecular target for tumour therapies include preferential expression in the tumours, a role in the development of the tumours (rather than merely being a marker), and a knowledge of the mechanism of its action as a starting point for the rational design of activity modulators. It appears that all of these three criteria are satisfied, at least to some extent, in the case of c-erbB-2 and breast cancer and a considerable research effort is currently being directed to this molecule.

It has recently been found that the alkaloid K252a, at appropriate concentrations, selectively inhibits the tyrosine kinase and biological activities of the trk-class of neurotrophin receptors, but has no effect on the activities of v-src, v-fms or the receptors for EGF and PDGF, and also does not affect general cell viability or proliferation (Tapley et al, Oncogene, 7, 371-381 (1992)). Members of another class of molecules, the tyrphostins, may be selective inhibitors of the EGFr/c-erbB-2 class of tyrosine kinases (Gazit et al, J. Med. Chem., 34, 1896-1907 (1991)). An antibody which inhibits the proliferation of human tumour cells by binding to the c-erbB-2 protein is currently under evaluation as a potential cancer therapy (Carter et al, Proc. Natl. Acad. Sci. (USA), 89, 4285-4289 (1992)).

If the transformation of a significant minority of breast tumour cells is due, at least in part, to the dysregulated activity of the c-erbB-2 tyrosine kinase, then it seems likely that other breast tumours owe aspects of their transformed phenotype to the activity of other tyrosine kinases. The identification of further tumour associated tyrosine kinases would allow the development of novel diagnostic and therapeutic approaches to cancer and in particular breast tumours.

10

The cDNA cloning of a previously novel receptor tyrosine kinase from breast carcinoma cells has recently been reported (Johnson et al., Proc. Natl. Acad. Sci. USA, 90, 5679-5681 (June 1993)) and the DNA and predicted amino acid sequence of this protein are set out in

15

SEQ ID NO. 1.

The predicted amino acid sequence is set out separately in

20

SEQ ID NO. 2.

The authors designated this protein tyrosine kinase DDR (Discoidin domain receptor) and detected the transcription product of the DNA sequence in other human breast carcinoma cell lines and the translation product (protein) was also detected in breast carcinoma cell lines. However, the authors do not suggest that the protein tyrosine kinase plays any role as a tumour causative agent and no potential utility is suggested in the therapy or diagnosis of cancer, in particular human breast cancer.

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30

Another recent disclosure suggests that the same protein tyrosine kinase has been detected in an epithelial ovarian cancer cell line (Laval et al, American Association for Cancer Research, Meeting, May 1993, Abstract No. 3144).

35

It has now been found that the receptor tyrosine kinase referred to above is expressed at a much higher level in human breast tumour cells than in normal breast. This suggests an

involvement of the tyrosine kinase in the transformation of breast cells. The present invention relates to the use of the tyrosine kinase as defined above in the diagnosis and/or therapy of cancer, particularly human breast tumours.

5

The present invention involves the use of a DNA isolate encoding a protein tyrosine kinase having the amino acid sequence shown in

SEQ ID NO. 1

10 or an amino acid sequence showing a significant degree of homology thereto, preferably at least 60% homology in the catalytic domain and at least 40% homology in the remainder of the molecule, more preferably at least 90% homology overall, for example at least 95% homology, or a fragment
15 thereof.

According to one embodiment, the DNA isolate encodes all of the amino acid sequence shown in the SEQ ID NO. 1 or an amino acid sequence showing a significant degree of homology
20 thereto. According to another embodiment the DNA isolate encodes a fragment of the amino acid sequence of the protein tyrosine kinase or an amino acid sequence showing a significant degree of homology thereto. DNA sequence encoding fragments of the protein tyrosine kinase preferably encodes
25 those parts of the amino acid sequence which characterise the enzyme, i.e. those parts which are most distinct from other protein tyrosine kinases. Most preferably the DNA sequence encodes all or part of the catalytic domain of the enzyme, i.e. amino acid residues 617 to 907 as shown in SEQ ID NO. 1.

30

The DNA isolate may have the base sequence defined in SEQ ID NO. 1 to encode the relevant amino acid sequence. Alternatively the DNA isolate may have any other DNA sequence encoding the relevant amino acid sequence.

35

The DNA isolate may take the form of a cloning vector or an expression vector preferably a plasmid vector, including DNA as defined above. In the case of an expression vector the DNA will be under control of an appropriate promoter and will

include regulatory elements required for expression in a suitable host cell.

5 The present invention also involves the use of a recombinant cell line transformed with an expression vector as defined above and capable of expressing a recombinant protein tyrosine kinase having amino acid sequence shown in SEQ ID NO. 1
or an amino acid sequence showing a significant degree of
10 homology thereto or a fragment thereof.

Suitable host cells include mammalian cells, insect cells, yeast cells and bacterial cells and the expression vector will include a promoter and other regulatory elements
15 appropriate to the host cell in question. Preferred host cells include CHO cells, myeloma cells, primary immortalised human breast cells, rodent fibroblast cell lines, baculovirus cells, yeast and *E. coli* cells. General techniques for manipulating DNA coding sequences and expressing such
20 sequences in various types of cells are well known to those skilled in the art and are described for example in Sambrook et al, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory.

25 The present invention also involves the use of a protein tyrosine kinase having the amino acid sequence shown in SEQ ID NO. 1
or an amino acid sequence showing a significant degree of
homology thereto or a fragment thereof.

30 The protein tyrosine kinase or a fragment thereof may be a recombinant polypeptide produced as the expression product of a coding sequence as defined above in a recombinant cell line. Alternatively the protein tyrosine kinase may be
35 produced in a human tumour cell line. In either case the protein may be extracted and purified by standard techniques, for example antibody affinity chromatography. The glycosylation of the protein (if any) will depend on the cells in which it is produced and protein produced in human tumour

cells in culture should have glycosylation equivalent to that produced *in vivo* in human tumours.

Part of the DNA sequence encoding the protein tyrosine kinase set out in SEQ ID NO. 1 was isolated in the manner described in more detail in the example set out below but which involves briefly the following steps:

- i) isolation of mRNA from tumour metastatic tissue;
- ii) preparation of cDNA from the isolated mRNA;
- 10 iii) PCR amplification using degenerate oligonucleotide primers designed to amplify sequence associated with protein tyrosine kinases;
- iv) subcloning PCR products;
- v) identification of protein tyrosine kinase products
- 15 amplified at higher levels from tumour bearing tissue;
- vi) DNA sequencing and identification of novel protein tyrosine kinase sequence;
- vii) isolation of cDNA sequence 3' of the region amplified by 3' RACE PCR.

20

Application of the above protocol identified part of the DNA sequence for the protein tyrosine kinase which was designated PTK22 and which corresponds to the protein tyrosine kinase designated DDR by Johnson et al (Supra). As already

25 noted, the enzyme has now been shown to be overexpressed in certain human breast tumours as compared to normal breast tissue.

The present invention thus relates to the application of

30 the tyrosine kinases for the development of therapeutic, prognostic and diagnostic approaches to cancer. The invention is particularly applicable to breast cancer, however, the same approaches may also be applicable to other cancers and research data suggests a connection between breast cancer and

35 ovarian cancer and also between these two cancers and gastric cancer.

In terms of therapy, the involvement of the tyrosine kinase in tumours means that beneficial clinical effects in

the treatment of tumours can be obtained by modulating the tyrosine kinase activity and/or the functionality of the receptor. There are a number of ways in which such modulation could be achieved.

5

The activity of the protein tyrosine kinase could be significantly impaired or inhibited by small chemical molecules and screens to identify suitable small molecular weight inhibitors can be developed as described in more detail below. This approach is analogous to that proposed for other tyrosine kinases (see "Drugs of the Future", 17(2), 119-131, (1992)). A further embodiment of this approach would be to derive nmr spectra or crystal structures for the protein tyrosine kinases or domains thereof and use the structural information so obtained to synthesise chemical structures de novo which could be similarly screened for activity as inhibitors.

The approach described above could be extended to encompass small peptides which are either competitive for signalling action of the tyrosine kinase receptor, or which demonstrate useful binding thereto, such that the receptor function is inhibited. This would include, for example, peptides which are capable of blocking substrate binding to the receptor, including but not limited to those which bear SH2 domains. An additional approach is to use small molecules or peptides to block or interfere with ligand activation or dimerisation of the tyrosine kinase. Both of these events are generally required to effect a biological signal so that blocking or inhibition thereof could be used to therapeutic effect.

Macromolecules, such as antibodies, could also be used as inhibitory molecules to block receptor function. This would be particularly applicable to antibodies raised against the extracellular domain of the protein tyrosine kinase where antibodies could promote internalisation or interfere with ligand binding. Either of these situations could produce significant modulation (positive or negative) of the activity

of the protein.

Protein tyrosine kinase function could also be blocked by expressing or administering a peptide which is a truncated
5 or altered version of the protein. These are typically referred to as dominant negative proteins and are believed to sequester the active protein in a non-functional complex. Protein tyrosine kinase function could also be modulated by blocking translation of the mRNA encoding the protein using
10 antisense oligonucleotides.

A further regulatory role of the protein tyrosine kinase could be manipulated by controlling expression or activity of specific phosphatases which control the degree of protein
15 phosphorylation. It is recognised that the phosphorylation of specific residues, particularly tyrosine, threonine and serine residues, play an important role in transmitting a biological signal from the tyrosine kinase.

20 Use of the above therapeutic approaches depends on the identification and/or development of suitable agents for modulating, for example inhibiting, particular functions or activities of the protein tyrosine kinase. The present invention thus relates to assays or screens by which such
25 agents can be identified.

The present invention provides a method of screening a substance for potential utility as a therapeutic agent in the treatment of cancer, in particular breast cancer, which
30 comprises providing a standard system in which a protein tyrosine kinase or an active fragment thereof is able to develop a measurable effect, allowing the protein tyrosine kinase to develop that effect in the presence and absence of the said substance and measuring that effect, ability to
35 produce significant inhibition of the effect being taken as an indication of potential utility as a therapeutic agent, wherein the protein tyrosine kinase is characterised by the amino acid sequence shown in

SEQ ID NO. 1

or an amino acid sequence showing a significant degree of homology thereto.

In accordance with this general principle, screens for potential therapeutic agents can take a number of forms. For example an initial screen to determine whether or not a substance merits further investigation as a potential inhibitor of the protein tyrosine kinase will usually be biochemical and should preferably be simple, rapid and capable of high through-put. Such screens will often make use of the protein expressed in a recombinant expression system or derived from a cellular source in which the protein is overexpressed. Particularly suitable recombinant expression systems include insect baculovirus, and the use of primary immortalised human breast cell lines, or rodent fibroblast cell lines as host cells. The protein is then used directly in an *in vitro* assay with and without the potential inhibitor. This gives rapid data on the performance of the substance as an inhibitor often providing direct evidence that the substance can inhibit the enzymic activity of the protein tyrosine kinase and thus merits further study. This type of isolated system can also provide data on specificity but will not generally provide any information on bioavailability. The enzymic activity of the protein tyrosine kinase in vitro can be measured, for example, by measuring autophosphorylation or phosphorylation of a model substrate, by following incorporation of radioactive phosphate or by using anti-phosphotyrosine antibodies.

Further information concerning potential inhibitors can be provided by cell based screens which make use of a phenotypic alteration, e.g. a change in morphology and/or tumorigenicity, conferred by expression of the protein tyrosine kinase in a recombinant cell line or overexpression in any other available cell line. Particularly suitable recombinant cell lines again use primary immortalised human breast cell lines or rodent fibroblast cell lines as host cells. The cells can be used in proliferation or tumorigenicity assays with and without the potential inhibitor

looking for substances able to halt growth of and/or morphologically de-transform the cells. A control for substances which are generally cytotoxic can be provided by the same parental cell line engineered to express another
5 oncogene which transforms the cells at a signalling point downstream of the protein tyrosine kinase. Assays of this sort can be very informative and they may provide data on mode of action of inhibitors.

10 The final stage of screening is the development of animal screens. These are time consuming and expensive and for these and ethical reasons their use is kept to a minimum, however they can give vital information regarding metabolism, clearance and performance of a substance in a true *in vivo*
15 system which cannot be obtained in other ways. Animal screens ideally use the same recombinant or other cell lines as described above which express the protein tyrosine kinase. The cells are used as xenografts in animals, for example, nude mice, to give tumours whose growth is controlled by expression
20 of the protein tyrosine kinase. The mice are then given inhibitors to determine whether they are capable of causing the selective regression of the xenografts without blocking the growth of a control xenograft. Alternatively transgenic animals which develop tumours by virtue of the appropriate
25 tissue specific expression of the target protein tyrosine kinase can be used in a similar manner.

The invention also extends to therapeutic agents identified by use of any or all of the screens referred to
30 above. Preferably the therapeutic agent is a chemical molecule of relatively low molecular weight, for example, less than about 1000. Examples of suitable classes of molecule include staurosporine analogues, tyrphostins and flavonoids. Alternatively, the therapeutic agent can be a macromolecule,
35 for example an antibody raised against the extracellular domain of the protein tyrosine kinase. The therapeutic agent can also be a peptide or an antisense oligonucleotide capable of blocking translation of the mRNA encoding the protein tyrosine kinase.

The invention also extends to an enzyme-substrate complex which comprises a protein tyrosine kinase characterised by the amino acid sequence shown in

SEQ ID NO. 1

- 5 or an amino acid sequence showing a significant degree of homology thereto and a therapeutic agent capable of modulating the activity of the said protein tyrosine kinase.

- 10 The protein tyrosine kinases can also be used in prognostic and diagnostic applications. Thus antibodies raised against the protein tyrosine kinase or nucleic acid encoding the tyrosine kinase can be used as the basis for screening tissue, in particular tumour tissue, for the presence of the protein tyrosine kinases. If, as in the case
15 of c-erbB-2, the expression of the tyrosine kinase correlates with poor prognosis, then treatment of the patient could be modified accordingly. Similarly, again as with c-erbB-2, there may be situations where parts of the receptor are "shed" into the bloodstream. In this case, the presence of the
20 receptor could be detected, for example, by use of an ELISA assay, and this would be a useful diagnostic tool for the identification of patients predisposed to develop or liable to develop tumours, in particular breast cancer.

- 25 Accordingly the present invention provides a method for detecting a tyrosine kinase having an amino acid sequence as defined in

SEQ ID NO. 1

- or a fragment thereof which comprises reacting a test sample
30 with a specific antibody raised against an antigen from the said amino acid sequence and determining whether there is any antigen-antibody binding within the test sample. The test sample may be for example a tissue sample, such as a tumour sample, or a blood sample and presence of abnormal amounts of
35 the protein tyrosine kinase may indicate development of or susceptibility to develop a tumour.

The invention also provides a method for the detection in a sample of DNA a nucleic acid sequence encoding a tyrosine

kinase which comprises subjecting the sample or nucleic acid isolated therefrom to a method capable of detecting a nucleic acid sequence therein identical or substantially homologous to a defined nucleic acid sequence and identifying any
5 sequence identical or substantially homologous to all or part of the DNA sequence defined in

SEQ ID NO. 1,

or mRNA which would be the transcription product of such DNA. Again, presence of abnormal amounts of nucleic acid encoding
10 the protein tyrosine kinase may indicate development of or susceptibility to develop a tumour.

Suitable methods for the detection of specific DNA sequences include Southern blotting and/or PCR using
15 appropriate primers. Suitable methods for detecting mRNA include Northern blotting, RNase protection studies and direct nucleic acid *in situ* hybridisation.

EXAMPLE

20

Experimental work on which the invention is based is described in more detail in this Example in which reference is made to the accompanying drawings in which:

25 Figure 1 shows a Northern blot to examine expression of DDR mRNA in human axillary lymph nodes. 5µg of total RNA were loaded per lane. Approximately 4 kb DDR transcript (upper panel). Ethidium bromide staining of 28S rRNA, to demonstrate RNA loading, is shown (bottom panel).

30

Figure 2 shows *in situ* hybridisation to examine DDR expression in an involved lymph node. Light and dark field images are on the left and right respectively. Specific probe (DDR) and control (Cont.) are shown. Oligonucleotide probes were used.

35

Magnification x 200.

Figure 3 shows a Northern blot to examine DDR expression in human breast tissue. 10µg of total RNA per lane. Upper panel shows DDR transcript, lower panel ethidium bromide stained 28S

rRNA.

Figure 4 shows DDR expression in murine mammary development. 2µg Poly (A)+ RNA per lane. DDR transcript shown in upper panel. Blot was hybridised with GAPDH to demonstrate RNA loading (lower panel).

Figure 5 shows Northern blot analysis of DDR expression in human cell lines performed as for Figure 1. DDR transcript (upper panel), ethidium bromide stained 28S rRNA (lower panel).

Figure 6 shows *in situ* hybridisation analysis of DDR expression in human breast tumour tissue. Tissues were from three different individuals: a, b and c. In each panel, light and dark field images are on the left and right respectively. Specific probes are shown (DDR), and controls (Cont.). il= infiltrating lymphocytes, tu= tumour and ne= normal epithelium. Experiments 6a and 6b used oligonucleotides probes, and 6c used RNA probes. Magnification x 200.

Figure 7 shows DDR expression in normal tissues. a) human multiple tissue Northern blot containing 2µg poly (A)+ RNA per lane. DDR transcript shown in upper panel. Blot was stripped and rehybridised with human GAPDH probe to demonstrate RNA loading (lower panel). b) Total RNA Northern blot, 10µg RNA per lane, DDR transcript upper panel, ethidium bromide stained 28S rRNA lower panel.

MATERIALS AND METHODS

Tissues

Human tumour bearing or non-involved lymph nodes, tumour and normal tissue samples were obtained from surgery and rapidly frozen in liquid nitrogen. Normal breast tissue was obtained from reduction mammoplasty. Murine mammary tissue was obtained by excision of the fourth gland, removal of the associated lymph node, and rapid freezing in liquid nitrogen. All tissues were stored at -70°C or in liquid nitrogen.

Histopathological examination determined the presence or absence of metastases in the lymph nodes.

Cell lines

- 5 CAL 51, a human breast carcinoma cell line, was isolated and described by Gioanni et al (Br. J. Cancer, 62, 8-13 (1990)). The fibrosarcoma cell line HT1080, and the other breast cancer cell lines were purchased from the American Type Culture Collection. Cells were cultured according to conditions
10 recommended by the suppliers.

RNA extraction

- Total RNA was extracted from all tissues and cell lines by the method of Chomczynski and Sacchi (Anal. Biochem., 162, 156-159
15 (1987)). Poly (A)+ RNA was isolated from murine mammary gland total RNA samples using the Poly A+ Tract Kit (Promega) according to the manufacturer's instructions, except that mRNA was eluted with three washes (2 x 200 μ l, 1 x 100 μ l) of water.

20 RT-PCR and PTK differential screen

- 5 μ g of total RNA purified from involved or non-involved lymph nodes was digested with 10 units of RNase free DNase 1 (Pharmacia) using the manufacturer's recommended conditions in the presence of 40 units of RNase inhibitor (Boehringer
25 Mannheim). Samples were heated to 100°C for 1 minute (min) to inactivate the DNase, extracted with phenol/chloroform and precipitated with ethanol. For cDNA synthesis, 2.5 μ g of RNA was resuspended in water and denatured at 65°C for 3 min in the presence of 40 ng pd(N)₆ random hexamers (Pharmacia) and
30 cooled on ice. Reverse transcription was carried out using 1 mM dNTPs (Pharmacia), 40 units RNase inhibitor, 30 units of AMV reverse transcriptase SL (Life Sciences) and the buffer provided by the manufacturer at 41°C for 1 hour. Control reactions were performed as above but omitting the reverse
35 transcriptase enzyme (RT-control). To inactivate the inhibitory properties of reverse transcriptase on Taq polymerase, samples were heated to 100°C for 1 min.

Degenerate oligonucleotides were designed to amplify by PCT

an approximately 200 bp region encoding part of the catalytic domain of PTKs. The highly conserved amino acid sequence shown in

SEQ ID NO. 3

5 was used for designing the 5' oligonucleotide shown in

SEQ ID NO. 4

and the highly conserved amino acid sequence shown in

SEQ ID NO. 5

was used for designing the 3' oligonucleotide shown in

10 SEQ ID NO. 6.

Amplifications were carried out using cDNA derived from 0.5µg of original RNA in 60 mM KCl, 15mM Tris-HCl, 1.1mM MgCl₂, 200µM of each dNTP, 0.5ng of each primer and 2 units of Taq polymerase (Cetus). The reaction mixtures were cycled 15 times at 94°C for 1 min, 55°C for 2 min and 72°C for 1 min. For each sample, PCR was carried out on cDNA and the relevant RT-control. The amplified products were electrophoresed through 3% agarose gels, the approximately 200 bp band excised and purified using a Mermaid kit (Bio 101). PCR products from 20 involved nodes were subcloned into the pCR II vector (Invitrogen) according to the manufacturer's instructions. Clones were picked, grown in liquid culture, pelleted, resuspended in denaturing solution (0.5M NaOH, 1.5M NaCl) and aliquots were gridded onto duplicate Hybond-N membranes 25 (Amersham). Filters were then neutralised in 3M NaCl, 0.5M Tris-HCl (pH 7.4). Duplicate filters were hybridised with ³²P labelled randomly primed (Feinberg & Vogelstein, Anal. Biochem, 132, 6-13 (1983); and Feinberg & Vogelstein, Anal. Biochem, 137, 266-267 (1984)) PCR products from either the 30 involved or non-involved node. Clones containing PTK fragments that hybridised more strongly with the involved node-derived probe compared with the non-involved node derived probe were chosen for sequence analysis.

35 DNA sequencing

Templates for sequencing were prepared by the rapid method of Yie et al (Nucleic Acids Res., 21, 361(1993)) and were sequenced by the dideoxy method of Sanger et al, (Proc. Natl. Acad. Sci. (USA), 74, 5463-5467 (1977)) using a Sequenase

Version 2.0 kit (United States Biochemicals).

3' RACE-PCR

To isolate cDNA sequences 3' to the region amplified by the
5 PTK PCR, a modification of the RACE protocol described by
Frohman et al (Proc. Natl. Acad. Sci. (USA), 85, 8998-9002
(1988)) was used. RNA from human breast carcinoma cell line
MDA MG 468 was reverse transcribed using the tagged oligo d(T)
primer shown in

10 SEQ ID NO. 7.

Two nested sense oligonucleotides were used in consecutive PCR
reactions. The upstream sense primer was as shown in

SEQ ID NO. 8

and the downstream sense primer was as shown in

15 SEQ ID NO. 9.

The tag sequence used with oligo d(T) for the reverse
transcription was used as the antisense primer in both
amplifications. The reaction buffer was as described for the
PTK PCR except $MgCl_2$ was used at 1.5mM, cycling parameters
20 were 94°C for 1 min 15 seconds, 56°C for 2 min and 72°C for
2 min.

Northern blots

Human total RNA samples were electrophoresed through
25 formaldehyde/agarose gels, transferred to Hybond-N membranes
(Amersham) and fixed by U.V. illumination. The human multiple
tissue Northern blot was purchased from Clontech. Murine Poly
(A)+ RNA samples were fractionated in agarose gels containing
glyoxal/DMSO, transferred to ZetaProbe membranes (Biorad) and
30 fixed by baking at 80°C for 2 hours. ^{32}P labelled probes were
prepared as described by Feinberg & Vogelstein (supra).
Hybridisations were carried out according to the
manufacturer's instructions, membranes were washed to a final
stringency of 0.1 x SSC, 0.1% SDS at 65°C.

35

Probe generation for in situ hybridisation

Riboprobes: The DDR RACE PCR fragment was sub-cloned into
pBluescript KS⁺ (Stratagene) and sense and antisense RNAs
labelled with ^{35}S -UTP (Amersham) were synthesised. The

transcription reactions were carried out using a Stratagene Transcription kit according to the manufacturer's instruction. Probes were subject to limited alkaline hydrolysis at 65°C for 100 min (Cox et al., Developmental Biology, 101, 485-502 (1984)) to achieve a final length of between 100-200 bases. Full length and hydrolysed probes were examined on formaldehyde/agarose gels.

Oligonucleotide probes: Antisense 30mer oligonucleotides as follows were synthesised to the 3' untranslated and catalytic domain encoding regions of DDR:-

- SEQ ID NO. 10
- SEQ ID NO. 11
- SEQ ID NO. 12
- SEQ ID NO. 13.

A 30mer random oligonucleotide N₃₀ was also synthesised (randomer).

The oligonucleotides were 3'-labelled with ³³P-dATP (Amersham) using terminal transferase according to manufacturer's instructions (Boehringer Mannheim). Probes were purified by passing through Chroma-spin 10 columns (Clontech) to remove unincorporated label. Tail lengths were analysed on a DNA sequencing gel. The four specific oligonucleotides were then mixed equally to form a cocktail.

Labelling efficiency of all probes was assessed by scintillation counting.

In situ hybridisation

Except those used in post-hybridisation steps, all solutions were diethylpyrocarbonate treated and all glassware was baked.

7µm cryostat sections were cut from snap frozen human tumour material which had been excised during surgery, and stored in either liquid nitrogen or at -80°C. Sections were dried onto APTES (3-aminopropyltriethoxysilane) treated slides (Rentrop et al., Histochem. J., 18, 271-276 (1986)) over dry ice, and fixed at 4°C in 4% paraformaldehyde, phosphate buffered saline

(PBS) for 15 min, followed by two 15 min washes in PBS. The sections were dehydrated through ethanols from 30%-100%, air dried, and stored at -80°C until required.

5 a) With riboprobes

Sections were equilibrated to room temperature for 5 min, quickly rehydrated through ethanols from 100% to 30%, and washed twice for 5 min in PBS. Sections were acetylated for
10 10 min in 0.1M triethanolamine, 0.25% acetic anhydride while stirring. After washing in 2xSSC for 2 min, denaturation of tissue RNAs was carried out in 50% formamide, 2xSSC for 10 min at 60°C. Sections were then dehydrated and air dried.

15 Hybridisation was performed overnight in buffer (50% formamide, 0.3M NaCl, 10mM Tris- HCl pH8, 2mM EDTA pH8, 10% dextran sulphate, 10mM sodium phosphate pH8, 50µgml⁻¹ssDNA) with comparable counts per minute (cpm) of either antisense or sense riboprobes, in a humid chamber at 50°C.

20 After hybridisation, two 30 min washes in 2xSSC, 50% formamide at 60°C were followed by treatment with 20µgml⁻¹ RNase A in 0.5M NaCl, 10mM Tris HCl pH7.5, 5mM EDTA pH 7.5 for 30 min at 37°C. Sections were washed for 15 min in 2xSSC at 60°C,
25 followed by two final washes at a stringency of 0.1xSSC for 30 min at 60°C. Sections were dehydrated and air dried before autoradiography.

b) With oligonucleotide probes.

30 Sections were equilibrated to room temperature, rehydrated, washed and acetylated as described for sections hybridised with riboprobes. After washing twice for 5 min with PBS they were dehydrated and air dried.

35 Sections were overlaid with 10µl of hybridisation buffer (6x SSC, 50% formamide, 5x Denhardt's solution (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Edn., Cold Spring Harbor Laboratory Press, USA (1989)), 10% dextran sulphate)

containing $20\mu\text{gml}^{-1}$ unlabelled random oligonucleotide, and prehybridised for 3 hours at 37°C in a sealed humid chamber. After prehybridisation, a further $10\mu\text{l}$ of hybridisation buffer containing comparable cpm of either the labelled oligonucleotide cocktail or labelled randomer were added to each section and gently mixed with prehybridisation solution. The sections were incubated overnight at 37°C (Lathe, *In Situ Hybridisation Principles and Practice*, Polak & McGee (eds.) Oxford Scientific Publications, Oxford pp71-80 (1989)) in a sealed humid chamber.

Sections were removed from the chamber, and the hybridisation solution was rinsed off with prewarmed $5\times\text{SSC}$ gently pipetted over the slide. The area around the sections was dried and the sections were then overlaid with $20\mu\text{l}$ prehybridisation solution ($20\mu\text{gml}^{-1}$ randomer) and incubated for 60 min as before. This process might help reduce background by replacing non-specifically bound oligonucleotide with random oligonucleotide. This step was repeated once. Slides were washed to a final stringency of 60% formamide, $5\times\text{SSC}$ at 37°C . Sections were dehydrated in 70% and 100% ethanol and air dried.

Autoradiography on tissues

This process was the same for both riboprobe and oligonucleotide methods. In order to give a guideline of the length of time of exposure to photographic emulsion required, slides were placed against autoradiography film overnight. Slides were dipped in K.5 (Ilford) photographic emulsion diluted 1:1 with 2% glycerol, following manufacturer's guidelines. Slides were dried in an air-tight container with desiccant at room temperature for 2 hours and stored dry at 4°C , in a light-tight box for the required length of time. Slides were developed in D19 (Kodak), stopped in 1% acetic acid, 1% glycerol and fixed in 30% sodium thiosulphate. Sections were counter stained in 0.02% toluidine blue, mounted in DPX (Fisons) and examined by light and darkfield microscopy.

RESULTS

Identification of PTKs in a differential screen of involved and non-involved lymph nodes

5 RNA was isolated from involved and non-involved axillary lymph nodes from patients with metastatic breast tumours. RT-PCR was performed on the RNA using degenerate oligonucleotides encoding the amino acid sequences shown in SEQ ID NO. 3 and SEQ ID NO 5, sequences highly conserved in PTKs (Hanks,
10 Science, 241, 42-52 (1988)). The resultant pools of approximately 200bp PTK cDNA fragments from the involved nodes were subcloned, and clones screened with mixed pools of radiolabelled PTK fragments from both involved and non-involved nodes. Clones amplified preferentially from the
15 involved nodes were sequenced. For each of five involved lymph nodes, 50 or 100 clones were screened; differentially amplified cDNAs were found in four of five nodes. A novel kinase was detected as the major differentially amplified PTK-encoding cDNA; it showed similarities with trk A (Martin-Zanca
20 et al, Nature, 319, 743-748 (1986)) and the rat PCR generated fragment tyro 10 (Lai & Lemke, Neuron, 6, 691-704 (1991)). The receptor PTK c-erb-B2 was found at high levels in three nodes.

25 The trk A/tyro 10-like PTK is identical to DDR
To study the expression patterns of the trk-like kinase, a 3' Rapid Amplification of cDNA Ends (RACE)-PCR fragment was generated using two specific nested oligonucleotides corresponding to the sequence between the two degenerate
30 primers. Dideoxy sequencing of this 1.2 kilo base (kb) 3' RACE-PCR fragment revealed 384 nucleotides of predicted coding sequence. This comprised sequences encoding part of a putative PTK catalytic domain, which showed similarity to trk A, followed by a short C terminal domain of 8 amino acids
35 after subdomain XI. While analysing this fragment, a report was published describing a receptor PTK found in cultured breast carcinoma cells lines with an extracellular discoidin 1-like domain (DDR) (Johnson et al, Proc. Natl. Acad. Sci. (USA), 90, 5677-5681 (1993)). Comparison of the DDR sequence

with the 3' RACE-PCR fragment revealed them to be identical, the 3' RACE-PCR fragment begins at nucleotide position 2500 of the published DDR sequence.

5 **Expression of DDR mRNA in lymph nodes**

DDR was detected in a PCR based differential screen designed to identity PTKs expressed at higher levels in tumour containing compared with tumour free lymph nodes. In order to examine whether higher levels of DDR mRNA were present in the involved compared with the non-involved lymph node, the 3' RACE-PCR fragment radiolabelled with ³²P was used as a probe to hybridise with Northern blots of lymph node RNA. This probe recognised a single band of approximately 4kb on Northern blots. DDR mRNA was undetectable in the non-involved lymph node and present in four of six metastasis-containing nodes (Figure 1). *In situ* hybridisation on a section of involved lymph node showed specific hybridisation over the metastasised tumour cells (Figure 2).

20 **Expression of DDR mRNA in breast tumours, breast carcinoma cell lines and mammary development**

The expression patterns of DDR in a panel of breast tumour RNAs and a number of breast carcinoma cell lines were examined by hybridising Northern blots with the ³²P labelled 3' RACE-PCR fragment. DDR mRNA was present in normal breast tissue and in all the breast tumours tested (figure 3). Elevated levels of expression were detected in samples of 'normal' breast and carcinoma, both surgically removed from a pregnant woman. To examine DDR expression in mammary gland development, a Northern blot of murine mammary gland poly (A)+ RNA was hybridised with the 3' RACE-PCR derived probe. Figure 4 shows that murine DDR mRNA (NEP, Zerlin *et al*, Oncogene, 8, 2731-2739 (1983)), see discussion) was detected in all stages of development. Expression was moderate in virgin glands, a slight increase was seen in mid-pregnancy, during lactation very low levels of DDR mRNA were detected, and moderate levels reappeared during involution. During lactation casein mRNA comprises a large proportion of the total, this effectively dilutes the levels of other mRNAs; glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) mRNA levels were decreased in these samples also. DDR mRNA expression was also detected in fat pad samples. The fat pad represents mammary tissue which has developed in the absence of epithelial cells due to the cauterisation of the nipple and surrounding tissue prior to epithelial outgrowth. Varying amounts of DDR mRNA were detected in the breast cancer cell lines examined, the highest were in MCF 7 and T47D and the lowest were in MDA MB 157 and BT 474. Expression was not detected in the fibrosarcoma cell line HT1080 (Figure 5).

Northern blot analysis revealed that DDR mRNA was present in normal breast and breast tumour tissue. *In situ* hybridisation was, therefore, performed on normal breast tissue and primary breast carcinoma samples to determine which cell types express DDR. In sections from three tumour samples, (Figures 6 a, b and c) hybridisation was predominantly over the tumour cells. DDR mRNA was undetectable in normal breast tissue adjacent to the tumour (Figure 6c top panel), and in all normal breast samples examined (data not shown). Figure 6c (top panel), shows a mass of invasive carcinoma surrounded by stromal cells in the top right of the field, with normal epithelial tissue in the bottom left of the field; hybridisation was specifically over the tumour cells, and DDR mRNA was undetectable in the normal epithelium and stromal cells. Figure 6c (middle panel) shows DDR positive cancer cells which have replaced the normal epithelial cells in the breast lobules. The malignant epithelial elements are surrounded by a dense lymphocytic infiltrate in which DDR was undetectable. A comparable result to Figure 6c was obtained using oligonucleotide probes, (date not shown). Figures 6a and c (lower panels) show results obtained with the random oligonucleotide probe and the sense riboprobe respectively, with no specific hybridisation. These controls were carried out for all *in situ* hybridisations (not shown in all cases).

Patterns of DDR expression in normal human tissues

A human poly (A)⁺ RNA multiple tissue Northern blot was screened with the 3' RACE-PCR probe (Figure 7a). DDR was

expressed at high levels in kidney and placental tissue, low levels were detected in the heart and lung, and expression was undetectable in the liver. A more extensive panel of human tissues was examined on total RNA Northern blots (Figure 7b).

- 5 DDR was expressed in a wide range of tissues, with relatively high levels in the kidney cortex and thyroid. By this analysis, the haematopoietic tissues bone marrow and spleen did not have detectable levels of DDR mRNA.

10 DISCUSSION

- The initial differential screen identified DDR as a PTK expressed in involved but not in non-involved lymph nodes from breast cancer patients. Further studies revealed varying
- 15 levels of DDR mRNA in both normal breast and all of the breast carcinomas analysed. Elevated mRNA levels, relative to normal breast tissue, were found in both apparently normal breast tissue and breast carcinoma taken from the same pregnant patient. However, an examination of murine mammary gland
- 20 development revealed only a moderate increase in DDR mRNA in pregnancy. The higher levels of DDR in the human pregnant breast could be due to a focus of infiltrating carcinoma, as the specimen was taken from the tissue adjacent to a tumour. Alternatively, the murine mammary gland may differ from human
- 25 with respect to DDR expression during pregnancy. Unfortunately material was not available from the human pregnant breast for histopathological examination or *in situ* hybridisation studies to resolve this issue. *In situ* hybridisation studies were performed to determine which cells
- 30 in a particular tissue sample express DDR mRNA, and to give an indication of the relative abundance of mRNA expressed by different cell types. *In situ* hybridisation of a heavily infiltrated lymph node revealed DDR mRNA at high levels in the metastasising tumour cells and undetectable levels in the
- 35 adjacent cells. DDR mRNA was also shown to be present in tumour cells in primary breast carcinomas by *in situ* hybridisation. Where a direct comparison could be made, high levels of DDR mRNA were detected in the tumour cells but not in adjacent normal epithelial cells nor in tumour infiltrating

lymphocytes. By Northern analysis, DDR mRNA was present in normal human breast tissue, but by *in situ* hybridisation on human mammary tissue DDR has only been detected in tumour cells, which are epithelial in origin, and not in normal human breast epithelial or stromal cells. The question of the site of DDR expression in normal human mammary tissue is therefore unanswered; most likely cellular mRNA levels are too low for detection by *in situ* hybridisation. Nonetheless over expression of DDR occurs in some breast tumour cells when compared with normal mammary epithelium. Having demonstrated the presence of DDR mRNA in several sources of breast tumour tissue (in primary tumours, metastatic cells and carcinoma-derived cell lines) the expression in normal tissues was examined by Northern blotting. A wide range of levels were observed, ranging from high in the kidney cortex and thyroid, moderate in breast and lung, to undetectable amounts in the bone marrow, spleen, and liver. DDR expression is not restricted to any one tissue type, therefore, its role is not tissue specific. However, it is not ubiquitously expressed, thus a housekeeping function is unlikely and a cell specific role is probable.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: The Institute of Cancer Research
 (B) STREET: 17A, Onslow Gardens
 (C) CITY: London
 (E) COUNTRY: Great Britain
 (F) POSTAL CODE (ZIP): SW7 3AL

(A) NAME: The Wellcome Foundation Limited
 (B) STREET: Unicorn House, 160 Euston Road
 (C) CITY: London
 (E) COUNTRY: Great Britain
 (F) POSTAL CODE (ZIP): NW1 2BP

(ii) TITLE OF INVENTION: Cell Growth Factor Receptors

(iii) NUMBER OF SEQUENCES: 13

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3754 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 142..2883

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCCCGGGTCGG ACCGCCTGGG TCTGCCGGGA AGAGCGATGA GAGGTGTCTG AAGGTGGCTA	60
TTCACGTGAGC GATGGGGTTG GACTTGAAGG AATGCCAAGA GATGCTGCCC CCACCCCCTT	120
AGGCCCGAGG GATCAGGAGC T ATG GGA CCA GAG GCC CTG TCA TCT TTA CTG	171
Met Gly Pro Glu Ala Leu Ser Ser Leu Leu	
1 5 10	
CTG CTG CTC TTG GTG GCA AGT GGA GAT GCT GAC ATG AAG GGA CAT TTT	219
Leu Leu Leu Leu Val Ala Ser Gly Asp Ala Asp Met Lys Gly His Phe	
15 20 25	
GAT CCT GCC AAG TGC CGC TAT GCC CTG GGC ATG CAG GAC CGG ACC ATC	267
Asp Pro Ala Lys Cys Arg Tyr Ala Leu Gly Met Gln Asp Arg Thr Ile	
30 35 40	
CCA GAC AGT GAC ATC TCT GCT TCC AGC TCC TGG TCA GAT TCC ACT GCC	315
Pro Asp Ser Asp Ile Ser Ala Ser Ser Ser Trp Ser Asp Ser Thr Ala	
45 50 55	

	GCC CGC CAC AGC AGG TTG GAG AGC AGT GAC GGG GAT GGG GCC TGG TGC Ala Arg His Ser Arg Leu Glu Ser Ser Asp Gly Asp Gly Ala Trp Cys 60 65 70	363
5	CCC GCA GGG TCG GTG TTT CCC AAG GAG GAG GAG TAC TTG CAG GTG GAT Pro Ala Gly Ser Val Phe Pro Lys Glu Glu Glu Tyr Leu Gln Val Asp 75 80 85 90	411
10	CTA CAA CGA CTC CAC CTG GTG GCT CTG GTG GGC ACC CAG GGA CGG CAT Leu Gln Arg Leu His Leu Val Ala Leu Val Gly Thr Gln Gly Arg His 95 100 105	459
15	GCC GGG GGC CTG GGC AAG GAG TTC TCC CGG AGC TAC CGG CTG CGT TAC Ala Gly Gly Leu Gly Lys Glu Phe Ser Arg Ser Tyr Arg Leu Arg Tyr 110 115 120	507
20	TCC CGG GAT GGT CGC CGC TGG ATG GGC TGG AAG GAC CGC TGG GGT CAG Ser Arg Asp Gly Arg Arg Trp Met Gly Trp Lys Asp Arg Trp Gly Gln 125 130 135	555
	GAG GTG ATC TCA GGC AAT GAG GAC CCT GAG GGA GTG GTG CTG AAG GAC Glu Val Ile Ser Gly Asn Glu Asp Pro Glu Gly Val Val Leu Lys Asp 140 145 150	603
25	CTT GGG CCC CCC ATG GTT GCC CGA CTG GTT CGC TTC TAC CCC CGG GCT Leu Gly Pro Pro Met Val Ala Arg Leu Val Arg Phe Tyr Pro Arg Ala 155 160 165 170	651
30	GAC CGG GTC ATG AGC GTC TGT CTG CGG GTA GAG CTC TAT GGC TGC CTC Asp Arg Val Met Ser Val Cys Leu Arg Val Glu Leu Tyr Gly Cys Leu 175 180 185	699
35	TGG AGG GAT GGA CTC CTG TCT TAC ACC GCC CCT GTG GGG CAG ACA ATG Trp Arg Asp Gly Leu Leu Ser Tyr Thr Ala Pro Val Gly Gln Thr Met 190 195 200	747
40	TAT TTA TCT GAG GCC GTG TAC CTC AAC GAC TCC ACC TAT GAC GGA CAT Tyr Leu Ser Glu Ala Val Tyr Leu Asn Asp Ser Thr Tyr Asp Gly His 205 210 215	795
	ACC GTG GGC GGA CTG CAG TAT GGG GGT CTG GGC CAG CTG GCA GAT GGT Thr Val Gly Gly Leu Gln Tyr Gly Gly Leu Gly Gln Leu Ala Asp Gly 220 225 230	843
45	GTG GTG GGG CTG GAT GAC TTT AGG AAG AGT CAG GAG CTG CGG GTC TGG Val Val Gly Leu Asp Asp Phe Arg Lys Ser Gln Glu Leu Arg Val Trp 235 240 245 250	891
50	CCA GGC TAT GAC TAT GTG GGA TGG AGC AAC CAC AGC TTC TCC AGT GGC Pro Gly Tyr Asp Tyr Val Gly Trp Ser Asn His Ser Phe Ser Ser Gly 255 260 265	939
55	TAT GTG GAG ATG GAG TTT GAG TTT GAC CGG CTG AGG GCC TTC CAG GCT Tyr Val Glu Met Glu Phe Glu Phe Asp Arg Leu Arg Ala Phe Gln Ala 270 275 280	987
60	ATG CAG GTC CAC TGT AAC AAC ATG CAC ACG CTG GGA GCC CGT CTG CCT Met Gln Val His Cys Asn Asn Met His Thr Leu Gly Ala Arg Leu Pro 285 290 295	1035
	GGC GGG GTG GAA TGT CGC TTC CGG CGT GGC CCT GCC ATG GCC TGG GAG Gly Gly Val Glu Cys Arg Phe Arg Arg Gly Pro Ala Met Ala Trp Glu 300 305 310	1083
65	GGG GAG CCC ATG CGC CAC AAC CTA GGG GGC AAC CTG GGG GAC CCC AGA Gly Glu Pro Met Arg His Asn Leu Gly Gly Asn Leu Gly Asp Pro Arg 315 320 325 330	1131

	GCC CGG GCT GTC TCA GTG CCC CTT GGC GGC CGT GTG GCT CGC TTT CTG	1179
	Ala Arg Ala Val Ser Val Pro Leu Gly Gly Arg Val Ala Arg Phe Leu	
	335 340 345	
5	CAG TGC CGC TTC CTC TTT GCG GGG CCC TGG TTA CTC TTC AGC GAA ATC	1227
	Gln Cys Arg Phe Leu Phe Ala Gly Pro Trp Leu Leu Phe Ser Glu Ile	
	350 355 360	
10	TCC TTC ATC TCT GAT GTG GTG AAC AAT TCC TCT CCG GCA CTG GGA GGC	1275
	Ser Phe Ile Ser Asp Val Val Asn Asn Ser Ser Pro Ala Leu Gly Gly	
	365 370 375	
15	ACC TTC CCG CCA GCC CCC TGG TGG CCG CCT GGC CCA CCT CCC ACC AAC	1323
	Thr Phe Pro Pro Ala Pro Trp Trp Pro Pro Gly Pro Pro Pro Thr Asn	
	380 385 390	
20	TTC AGC AGC TTG GAG CTG GAG CCC AGA GGC CAG CCA AGG CCC GTG GCC	1371
	Phe Ser Ser Leu Glu Leu Glu Pro Arg Gly Gln Pro Arg Pro Val Ala	
	395 400 405 410	
	AAG GCC GAG GGG AGC CCG ACC GCC ATC CTC ATC GGC TGC CTG GTG GCC	1419
	Lys Ala Glu Gly Ser Pro Thr Ala Ile Leu Ile Gly Cys Leu Val Ala	
	415 420 425	
25	ATC ATC CTG CTC CTG CTG CTC ATC ATT GCC CTC ATG CTC TGG CGG CTG	1467
	Ile Ile Leu Leu Leu Leu Ile Ile Ala Leu Met Leu Trp Arg Leu	
	430 435 440	
30	CAC TGG CGC AGG CTC CTC AGC AAG GCT GAA CGG AGG GTG TTG GAA GAG	1515
	His Trp Arg Arg Leu Leu Ser Lys Ala Glu Arg Arg Val Leu Glu Glu	
	445 450 455	
35	GAG CTG ACG GTT CAC CTC TCT GTC CCT GGG GAC ACT ATC CTC ATC AAC	1563
	Glu Leu Thr Val His Leu Ser Val Pro Gly Asp Thr Ile Leu Ile Asn	
	460 465 470	
40	AAC CGC CCA GGT CCT AGA GAG CCA CCC CCG TAC CAG GAG CCC CGG CCT	1611
	Asn Arg Pro Gly Pro Arg Glu Pro Pro Pro Tyr Gln Glu Pro Arg Pro	
	475 480 485 490	
	CGT GGG AAT CCG CCC CAC TCC GCT CCC TGT GTC CCC AAT GGC TCT GCG	1659
	Arg Gly Asn Pro Pro His Ser Ala Pro Cys Val Pro Asn Gly Ser Ala	
	495 500 505	
45	TTG CTG CTC TCC AAT CCA GCC TAC CGC CTC CTT CTG GCC ACT TAC GCC	1707
	Leu Leu Leu Ser Asn Pro Ala Tyr Arg Leu Leu Leu Ala Thr Tyr Ala	
	510 515 520	
50	CGT CCC CCT CGA GGC CCG GGC CCC CCC ACA CCC GCC TGG GCC AAA CCC	1755
	Arg Pro Pro Arg Gly Pro Gly Pro Pro Thr Pro Ala Trp Ala Lys Pro	
	525 530 535	
55	ACC AAC ACC CAG GCC TAC AGT GGG GAC TAT ATG GAG CCT GAG AAG CCA	1803
	Thr Asn Thr Gln Ala Tyr Ser Gly Asp Tyr Met Glu Pro Glu Lys Pro	
	540 545 550	
60	GGC GCC CCG CTT CTG CCC CCA CCT CCC CAG AAC AGC GTC CCC CAT TAT	1851
	Gly Ala Pro Leu Leu Pro Pro Pro Pro Gln Asn Ser Val Pro His Tyr	
	555 560 565 570	
	GCC GAG GCT GAC ATT GTT ACC CTG CAG GGC GTC ACC GGG GGC AAC ACC	1899
	Ala Glu Ala Asp Ile Val Thr Leu Gln Gly Val Thr Gly Gly Asn Thr	
	575 580 585	
65	TAT GCT GTG CCT GCA CTG CCC CCA GGG GCA GTC GGG GAT GGG CCC CCC	1947
	Tyr Ala Val Pro Ala Leu Pro Pro Gly Ala Val Gly Asp Gly Pro Pro	
	590 595 600	

	AGA GTG GAT TTC CCT CGA TCT CGA CTC CGC TTC AAG GAG AAG CTT GGC Arg Val Asp Phe Pro Arg Ser Arg Leu Arg Phe Lys Glu Lys Leu Gly 605 610 615	1995
5	GAG GGC CAG TTT GGG GAG GTG CAC CTG TGT GAG GTC GAC AGC CCT CAA Glu Gly Gln Phe Gly Glu Val His Leu Cys Glu Val Asp Ser Pro Gln 620 625 630	2043
10	GAT CTG GTT AGT CTT GAT TTC CCC CTT AAT GTG CGT AAG GGA CAC CCT Asp Leu Val Ser Leu Asp Phe Pro Leu Asn Val Arg Lys Gly His Pro 635 640 645 650	2091
15	TTG CTG GTA GCT GTC AAG ATC TTA CGG CCA GAT GCC ACC AAG AAT GCC Leu Leu Val Ala Val Lys Ile Leu Arg Pro Asp Ala Thr Lys Asn Ala 655 660 665	2139
20	AGG AAT GAT TTC CTG AAA GAG GTG AAG ATC ATG TCG AGG CTC AAG GAC Arg Asn Asp Phe Leu Lys Glu Val Lys Ile Met Ser Arg Leu Lys Asp 670 675 680	2187
	CCA AAC ATC ATT CGG CTG CTG GGC GTG TGT GTG CAG GAC GAC CCC CTC Pro Asn Ile Ile Arg Leu Leu Gly Val Cys Val Gln Asp Asp Pro Leu 685 690 695	2235
25	TGC ATG ATT ACT GAC TAC ATG GAG AAC GGC GAC CTC AAC CAG TTC CTC Cys Met Ile Thr Asp Tyr Met Glu Asn Gly Asp Leu Asn Gln Phe Leu 700 705 710	2283
30	AGT GCC CAC CAG CTG GAG GAC AAG GCA GCC GAG GGG GCC CCT GGG GAC Ser Ala His Gln Leu Glu Asp Lys Ala Ala Glu Gly Ala Pro Gly Asp 715 720 725 730	2331
35	GGG CAG GCT GCG CAG GGG CCC ACC ATC AGC TAC CCA ATG CTG CTG CAT Gly Gln Ala Ala Gln Gly Pro Thr Ile Ser Tyr Pro Met Leu Leu His 735 740 745	2379
40	GTG GCA GCC CAG ATC GCC TCC GGC ATG CGC TAT CTG GCC ACA CTC AAC Val Ala Ala Gln Ile Ala Ser Gly Met Arg Tyr Leu Ala Thr Leu Asn 750 755 760	2427
	TTT GTA CAT CGG GAC CTG GCC ACG CGG AAC TGC CTA GTT GGG GAA AAT Phe Val His Arg Asp Leu Ala Thr Arg Asn Cys Leu Val Gly Glu Asn 765 770 775	2475
45	TTC ACC ATC AAA ATC GCA GAC TTT GGC ATG AGC CGG AAC CTC TAT GCT Phe Thr Ile Lys Ile Ala Asp Phe Gly Met Ser Arg Asn Leu Tyr Ala 780 785 790	2523
50	GGG GAC TAT TAC CGT GTG CAG GGC CGG GCA GTG CTG CCC ATC CGC TGG Gly Asp Tyr Tyr Arg Val Gln Gly Arg Ala Val Leu Pro Ile Arg Trp 795 800 805 810	2571
55	ATG GCC TGG GAG TGC ATC CTC ATG GGG AAG TTC ACG ACT GCG AGT GAC Met Ala Trp Glu Cys Ile Leu Met Gly Lys Phe Thr Thr Ala Ser Asp 815 820 825	2619
60	GTG TGG GCC TTT GGT GTG ACC CTG TGG GAG GTG CTG ATG CTC TGT AGG Val Trp Ala Phe Gly Val Thr Leu Trp Glu Val Leu Met Leu Cys Arg 830 835 840	2667
	GCC CAG CCC TTT GGG CAG CTC ACC GAC GAG CAG GTC ATC GAG AAC GCG Ala Gln Pro Phe Gly Gln Leu Thr Asp Glu Gln Val Ile Glu Asn Ala 845 850 855	2715
65	GGG GAG TTC TTC CGG GAC CAG GGC CGG CAG GTG TAC CTG TCC CGG CCG Gly Glu Phe Phe Arg Asp Gln Gly Arg Gln Val Tyr Leu Ser Arg Pro 860 865 870	2763

30

CCT GCC TGC CCG CAG GGC CTA TAT GAG CTG ATG CTT CGG TGC TGG AGC 2811
 Pro Ala Cys Pro Gln Gly Leu Tyr Glu Leu Met Leu Arg Cys Trp Ser
 875 880 885 890
 5 CGG GAG TCT GAG CAG CGA CCA CCC TTT TCC CAG CTG CAT CGG TTC CTG 2859
 Arg Glu Ser Glu Gln Arg Pro Pro Phe Ser Gln Leu His Arg Phe Leu
 895 900 905
 10 GCA GAG GAT GCA CTC AAC ACG GTG TGAATCACAC ATCCAGCTGC CCCTCCCTCA 2913
 Ala Glu Asp Ala Leu Asn Thr Val
 910
 GGGAGCGATC CAGGGGAAGC CAGTGACACT AAAACAAGAG GACACAATGG CACCTCTGCC 2973
 15 CCTTCCCTC CCGACAGCCC ATCACCTCTA ATAGAGGCAG TGAGACTGCA GGCTGGGCCC 3033
 ACCCAGGGAG CTGATGCCCC TTCTCCCTT CCTGGACACA CTCTCATGTC CCCTTCCTGT 3093
 TCTTCCTTCC TAGAAGCCCC TGTCGCCCAC CCAGCTGGTC CTGTGGATGG GATCCTCTCC 3153
 20 ACCCACCTCT AGCCATCCCT TGGGGAAGGG TGGGGAGAAA TATAGGATAG ACACTGGACA 3213
 TGGCCCATTG GAGCACCTGG GCCCCACTGG ACAACACTGA TTCCTGGACA GGTGGCTGCG 3273
 25 CCCCCAGCTT CTCTCTCCCT GTCACACACT GGACCCCACT GGCTGAGAAT CTGGGGGTGA 3333
 GGAGGACAAG AAGGAGAGGA AAATGTTTCC TTGTGCCTGC TCCTGTACTT GTCCTCAGCT 3393
 TGGGCTTCTT CCTCCTCCAT CACCTGAAAC ACTGGACCTG GGGGTAGCCC CGCCCCAGCC 3453
 30 CTCAGTCACC CCCCCTTCC CACCTGCAGT CTTGTAGCTA GAACTTCTCT AAGCCTATAC 3513
 GTTCTCTGTG AGTAAATATT GGGATTGGGG GGAAAGAGGG AGCAACGGCC CATAGCCTTG 3573
 35 GGGTTGGACA TCTCTAGTGT AGCTGCCACA TTGATTTTTC TATAATCACT TGGGTTTGTA 3633
 CATTTTTGGG GGGAGAGACA CAGATTTTTC CACTAATATA TGGACCTAGC TTGAGGCAAT 3693
 TTTAATCCCC TGCACTAGGC AGGTAATAAT AAAGGTTGAG TTTCCACAA AAAAAAAAAA 3753
 40 A 3754

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 914 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

55 Met Gly Pro Glu Ala Leu Ser Ser Leu Leu Leu Leu Leu Val Ala
 1 5 10 15
 Ser Gly Asp Ala Asp Met Lys Gly His Phe Asp Pro Ala Lys Cys Arg
 20 25 30
 60 Tyr Ala Leu Gly Met Gln Asp Arg Thr Ile Pro Asp Ser Asp Ile Ser
 35 40 45
 Ala Ser Ser Ser Trp Ser Asp Ser Thr Ala Ala Arg His Ser Arg Leu
 50 55 60
 65 Glu Ser Ser Asp Gly Asp Gly Ala Trp Cys Pro Ala Gly Ser Val Phe
 65 70 75 80

	Pro	Lys	Glu	Glu	Glu	Tyr	Leu	Gln	Val	Asp	Leu	Gln	Arg	Leu	His	Leu	
					85					90					95		
5	Val	Ala	Leu	Val	Gly	Thr	Gln	Gly	Arg	His	Ala	Gly	Gly	Leu	Gly	Lys	
				100					105					110			
	Glu	Phe	Ser	Arg	Ser	Tyr	Arg	Leu	Arg	Tyr	Ser	Arg	Asp	Gly	Arg	Arg	
			115					120					125				
10	Trp	Met	Gly	Trp	Lys	Asp	Arg	Trp	Gly	Gln	Glu	Val	Ile	Ser	Gly	Asn	
		130					135					140					
	Glu	Asp	Pro	Glu	Gly	Val	Val	Leu	Lys	Asp	Leu	Gly	Pro	Pro	Met	Val	
	145					150					155					160	
15	Ala	Arg	Leu	Val	Arg	Phe	Tyr	Pro	Arg	Ala	Asp	Arg	Val	Met	Ser	Val	
					165					170					175		
20	Cys	Leu	Arg	Val	Glu	Leu	Tyr	Gly	Cys	Leu	Trp	Arg	Asp	Gly	Leu	Leu	
				180					185					190			
	Ser	Tyr	Thr	Ala	Pro	Val	Gly	Gln	Thr	Met	Tyr	Leu	Ser	Glu	Ala	Val	
			195					200					205				
25	Tyr	Leu	Asn	Asp	Ser	Thr	Tyr	Asp	Gly	His	Thr	Val	Gly	Gly	Leu	Gln	
		210					215					220					
	Tyr	Gly	Gly	Leu	Gly	Gln	Leu	Ala	Asp	Gly	Val	Val	Gly	Leu	Asp	Asp	
	225					230					235					240	
30	Phe	Arg	Lys	Ser	Gln	Glu	Leu	Arg	Val	Trp	Pro	Gly	Tyr	Asp	Tyr	Val	
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35	Gly	Trp	Ser	Asn	His	Ser	Phe	Ser	Ser	Gly	Tyr	Val	Glu	Met	Glu	Phe	
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			275					280					285				
40	Asn	Met	His	Thr	Leu	Gly	Ala	Arg	Leu	Pro	Gly	Gly	Val	Glu	Cys	Arg	
		290					295					300					
	Phe	Arg	Arg	Gly	Pro	Ala	Met	Ala	Trp	Glu	Gly	Glu	Pro	Met	Arg	His	
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45	Asn	Leu	Gly	Gly	Asn	Leu	Gly	Asp	Pro	Arg	Ala	Arg	Ala	Val	Ser	Val	
					325					330					335		
50	Pro	Leu	Gly	Gly	Arg	Val	Ala	Arg	Phe	Leu	Gln	Cys	Arg	Phe	Leu	Phe	
				340					345					350			
	Ala	Gly	Pro	Trp	Leu	Leu	Phe	Ser	Glu	Ile	Ser	Phe	Ile	Ser	Asp	Val	
			355					360					365				
55	Val	Asn	Asn	Ser	Ser	Pro	Ala	Leu	Gly	Gly	Thr	Phe	Pro	Pro	Ala	Pro	
		370					375					380					
	Trp	Trp	Pro	Pro	Gly	Pro	Pro	Pro	Thr	Asn	Phe	Ser	Ser	Leu	Glu	Leu	
	385					390					395					400	
60	Glu	Pro	Arg	Gly	Gln	Pro	Arg	Pro	Val	Ala	Lys	Ala	Glu	Gly	Ser	Pro	
					405					410					415		
	Thr	Ala	Ile	Leu	Ile	Gly	Cys	Leu	Val	Ala	Ile	Ile	Leu	Leu	Leu	Leu	
				420					425					430			
65	Leu	Ile	Ile	Ala	Leu	Met	Leu	Trp	Arg	Leu	His	Trp	Arg	Arg	Leu	Leu	
				435				440					445				

	Ser	Lys	Ala	Glu	Arg	Arg	Val	Leu	Glu	Glu	Glu	Leu	Thr	Val	His	Leu
	450						455					460				
5	Ser	Val	Pro	Gly	Asp	Thr	Ile	Leu	Ile	Asn	Asn	Arg	Pro	Gly	Pro	Arg
	465					470				475						480
	Glu	Pro	Pro	Pro	Tyr	Gln	Glu	Pro	Arg	Pro	Arg	Gly	Asn	Pro	Pro	His
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15	Gly	Pro	Pro	Thr	Pro	Ala	Trp	Ala	Lys	Pro	Thr	Asn	Thr	Gln	Ala	Tyr
	530						535					540				
20	Ser	Gly	Asp	Tyr	Met	Glu	Pro	Glu	Lys	Pro	Gly	Ala	Pro	Leu	Leu	Pro
	545					550					555					560
	Pro	Pro	Pro	Gln	Asn	Ser	Val	Pro	His	Tyr	Ala	Glu	Ala	Asp	Ile	Val
				565						570					575	
25	Thr	Leu	Gln	Gly	Val	Thr	Gly	Gly	Asn	Thr	Tyr	Ala	Val	Pro	Ala	Leu
			580						585					590		
	Pro	Pro	Gly	Ala	Val	Gly	Asp	Gly	Pro	Pro	Arg	Val	Asp	Phe	Pro	Arg
			595					600					605			
30	Ser	Arg	Leu	Arg	Phe	Lys	Glu	Lys	Leu	Gly	Glu	Gly	Gln	Phe	Gly	Glu
	610						615					620				
	Val	His	Leu	Cys	Glu	Val	Asp	Ser	Pro	Gln	Asp	Leu	Val	Ser	Leu	Asp
35	625					630					635					640
	Phe	Pro	Leu	Asn	Val	Arg	Lys	Gly	His	Pro	Leu	Leu	Val	Ala	Val	Lys
				645						650					655	
40	Ile	Leu	Arg	Pro	Asp	Ala	Thr	Lys	Asn	Ala	Arg	Asn	Asp	Phe	Leu	Lys
				660					665					670		
	Glu	Val	Lys	Ile	Met	Ser	Arg	Leu	Lys	Asp	Pro	Asn	Ile	Ile	Arg	Leu
		675						680					685			
45	Leu	Gly	Val	Cys	Val	Gln	Asp	Asp	Pro	Leu	Cys	Met	Ile	Thr	Asp	Tyr
	690						695					700				
	Met	Glu	Asn	Gly	Asp	Leu	Asn	Gln	Phe	Leu	Ser	Ala	His	Gln	Leu	Glu
50	705					710					715					720
	Asp	Lys	Ala	Ala	Glu	Gly	Ala	Pro	Gly	Asp	Gly	Gln	Ala	Ala	Gln	Gly
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55	Pro	Thr	Ile	Ser	Tyr	Pro	Met	Leu	Leu	His	Val	Ala	Ala	Gln	Ile	Ala
				740					745					750		
	Ser	Gly	Met	Arg	Tyr	Leu	Ala	Thr	Leu	Asn	Phe	Val	His	Arg	Asp	Leu
			755					760					765			
60	Ala	Thr	Arg	Asn	Cys	Leu	Val	Gly	Glu	Asn	Phe	Thr	Ile	Lys	Ile	Ala
		770					775					780				
	Asp	Phe	Gly	Met	Ser	Arg	Asn	Leu	Tyr	Ala	Gly	Asp	Tyr	Tyr	Arg	Val
65	785					790					795					800
	Gln	Gly	Arg	Ala	Val	Leu	Pro	Ile	Arg	Trp	Met	Ala	Trp	Glu	Cys	Ile
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33

Leu Met Gly Lys Phe Thr Thr Ala Ser Asp Val Trp Ala Phe Gly Val
 820 825 830
 5 Thr Leu Trp Glu Val Leu Met Leu Cys Arg Ala Gln Pro Phe Gly Gln
 835 840 845
 Leu Thr Asp Glu Gln Val Ile Glu Asn Ala Gly Glu Phe Phe Arg Asp
 850 855 860
 10 Gln Gly Arg Gln Val Tyr Leu Ser Arg Pro Pro Ala Cys Pro Gln Gly
 865 870 875 880
 Leu Tyr Glu Leu Met Leu Arg Cys Trp Ser Arg Glu Ser Glu Gln Arg
 885 890 895
 15 Pro Pro Phe Ser Gln Leu His Arg Phe Leu Ala Glu Asp Ala Leu Asn
 900 905 910
 20 Thr Val

(2) INFORMATION FOR SEQ ID NO: 3:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 7 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

35 Arg Asp Leu Ala Ala Arg Asn
 1 5

(2) INFORMATION FOR SEQ ID NO: 4:

40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 32 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

50 GGAATTCTAG AMGSGACYTG GCVGCBAGRA AC

32

(2) INFORMATION FOR SEQ ID NO: 5:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- 10 (ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 5..one-of(2)
(D) OTHER INFORMATION: /note= "Xaa at position 5 is Tyr or Phe"
- 15 (ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 7..one-of(2)
(D) OTHER INFORMATION: /note= "Xaa at position 7 is Val or Ile"
- 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:
Asp Val Trp Ser Xaa Gly Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 6:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 35 (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

40 GGAATTCTAG ACACSCCRWA RSWCCASACR TC

32

(2) INFORMATION FOR SEQ ID NO: 7:

- 45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 50 (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

55 GACTCGAGTC GACATCGATT TTTTTTTTTT TTTT

35

35

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GTTGGGGAAA ATTTCACCAT C

21

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GGCATGAGCC GGAACCTC

18

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TCTCGATGAC CTGCTCGTCG GTGAGCTGCC

30

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CAGTCTCACT GCCTCTATTA GAGGTGATGG

30

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

AAGCTGAGGA CAAGTACAGG AGCAGGCACA

30

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TCAATGTGGC AGCTACACTA GAGATGTCCA

30

CLAIMS:

1. A method of screening a substance for potential utility as a therapeutic agent in the treatment of cancer which
5 comprises providing a standard system in which a protein tyrosine kinase or an active fragment thereof is able to develop a measurable effect, allowing the protein tyrosine kinase to develop that effect in the presence and absence of the said substance and measuring that effect, ability to
10 produce significant inhibition of the effect being taken as an indication of potential utility as a therapeutic agent, wherein the protein tyrosine kinase is characterised by the amino acid sequence shown in
SEQ ID NO. 1
15 or an amino acid sequence showing a significant degree of homology thereto.
2. A method as claimed in Claim 1 wherein the protein tyrosine kinase or an active fragment thereof is derived from
20 a recombinant expression system or a cellular source in which the protein is overexpressed.
3. A method as claimed in claim 2 wherein the recombinant expression system is an insect baculovirus, an immortalised
25 human breast cell line or a rodent fibroblast cell line.
4. A method as claimed in claim 2 or 3 wherein the measurable effect is protein tyrosine kinase activity which is measured by measuring autophosphorylation or
30 phosphorylation of a model substrate, by following incorporation of radioactive phosphate or by using anti-phosphotyrosine antibodies.
5. A method as claimed in Claim 1 wherein the measurable
35 effect is a phenotypic alteration conferred by expression of the protein tyrosine kinase or an active fragment thereof in a recombinant or non-recombinant cell line.

6. A method as claimed in Claim 1 wherein the measurable effect is the tumorigenic effect of a recombinant or non-recombinant cell line expressing the protein tyrosine kinase or an active fragment thereof when used as a xenograft in an animal.
7. A method as claimed in Claim 5 or 6 wherein the recombinant cell line is a primary immortalised human breast cell line or a rodent fibroblast cell line.
8. A therapeutic agent identified by a method as claimed in any of Claims 1 to 7.
9. A therapeutic agent as claimed in Claim 8 which is a chemical molecule of relatively low molecular weight.
10. A therapeutic agent as claimed in Claim 9 which is a staurosporine analogue, a tyrphostin or a flavenoid.
11. A therapeutic agent as claimed in Claim 8 which is a macromolecule.
12. A therapeutic agent as claimed in Claim 11 which is an antibody.
13. A therapeutic agent as claimed in claim 8 which is a peptide.
14. A therapeutic agent as claimed in claim 8 which is an antisense oligonucleotide capable of blocking translation of the mRNA encoding the protein tyrosine kinase.
15. A method for detecting a tyrosine kinase having an amino acid sequence as defined in
SEQ ID NO. 1
or a fragment thereof which comprises contacting a test sample with a specific antibody raised against an antigen from the

said amino acid sequence and determining whether there is any antigen antibody binding within the test sample.

16. A method for the detection in a sample of a nucleic acid sequence encoding a tyrosine kinase which comprises subjecting the sample or nucleic acid isolated therefrom to a method capable of detecting a nucleic acid sequence therein identical or substantially homologous to a defined nucleic acid sequence and identifying any sequence identical or substantially homologous to all or part of the DNA sequence defined in

SEQ ID NO. 1,
or mRNA which would be the transcription product of such DNA.

17. A method as claimed in Claim 16 wherein DNA is detected by Southern blotting and/or PCR using appropriate primers.

18. A method as claimed in Claim 16 wherein RNA is detected by Northern blotting, RNase protection studies and direct nucleic acid *in situ* hybridisation.

19. An enzyme-substrate complex which comprises a protein tyrosine kinase characterised by the amino acid sequence shown in

- SEQ ID NO. 1
or an amino acid sequence showing a significant degree of homology thereto and a therapeutic agent capable of modulating the activity of said protein tyrosine kinase.

20. A complex as claimed in Claim 19 wherein the therapeutic agent is a chemical molecule of relatively low molecular weight.

21. A complex as claimed in Claim 20 wherein the therapeutic agent is a staurosporine analogue, a tyrphostin or a flavenoid.

22. A complex as claimed in Claim 19 wherein the therapeutic

agent is a macromolecule.

23. A complex as claimed in Claim 22 wherein the therapeutic agent is an antibody.

5

24. A complex as claimed in Claim 19 wherein the therapeutic agent as a peptide.

10

25. A complex as claimed in Claim 19 wherein the therapeutic agent is an antisense oligonucleotide capable of blocking translation of the mRNA encoding the protein tyrosine kinase.

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FIG. 1

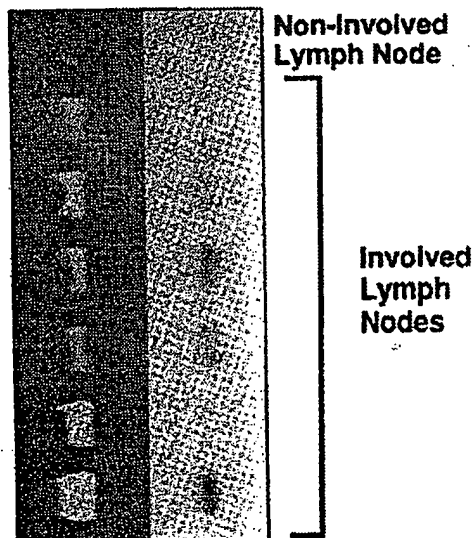
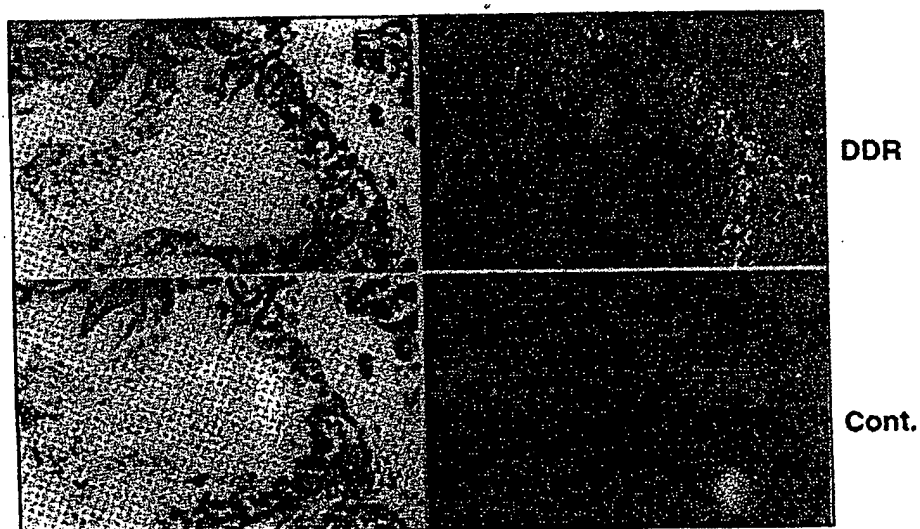


FIG. 2



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FIG. 4

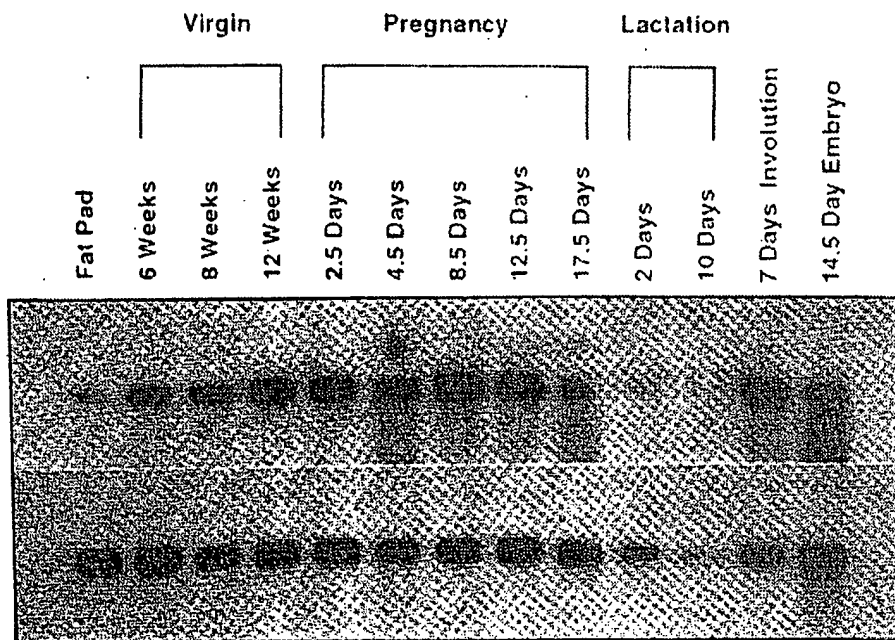
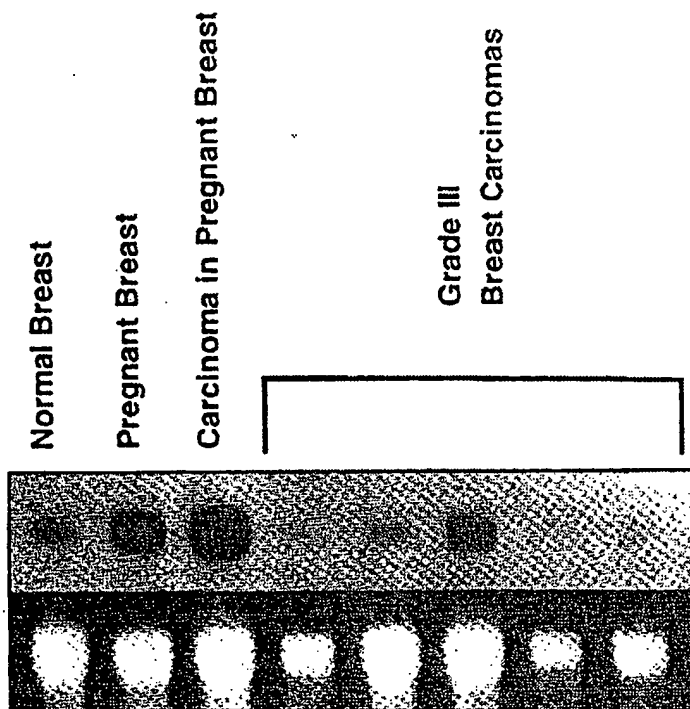


FIG. 3



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FIG. 5

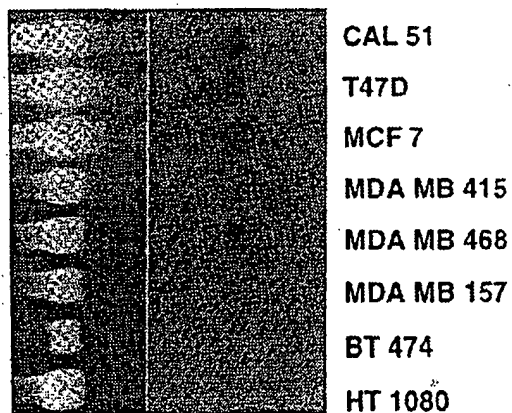
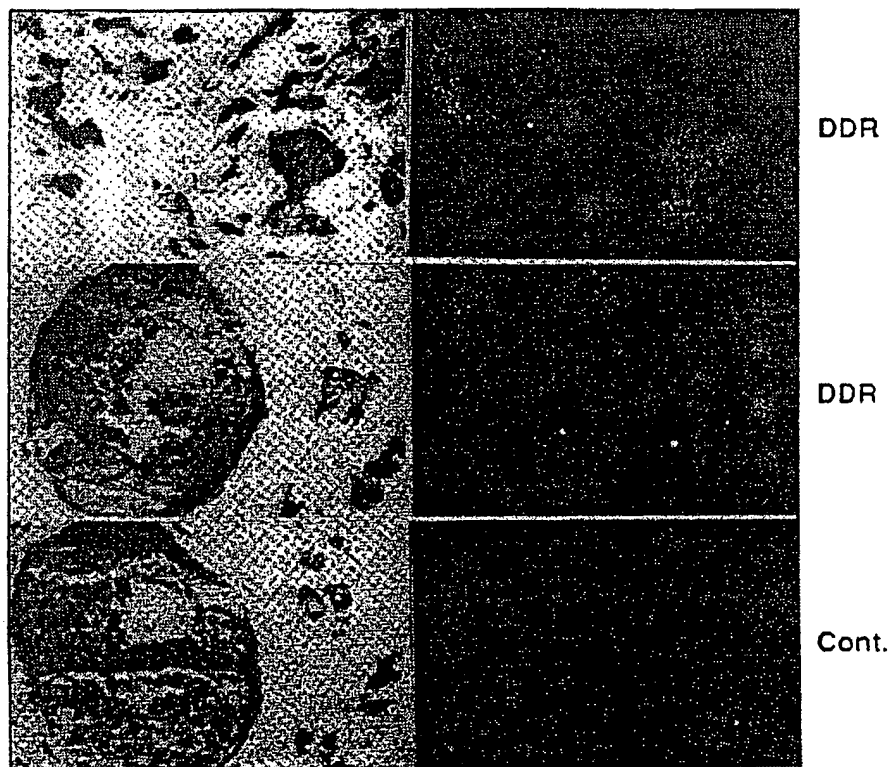


FIG. 6a



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FIG. 6b

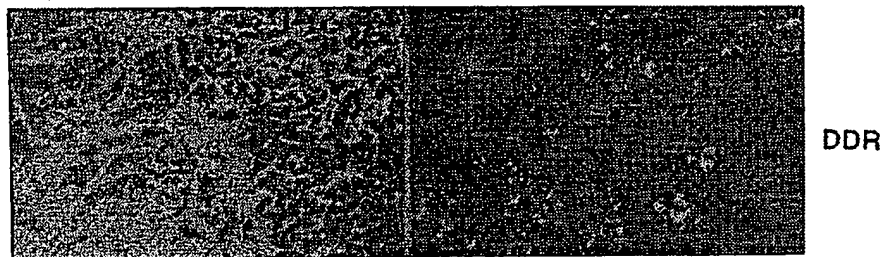
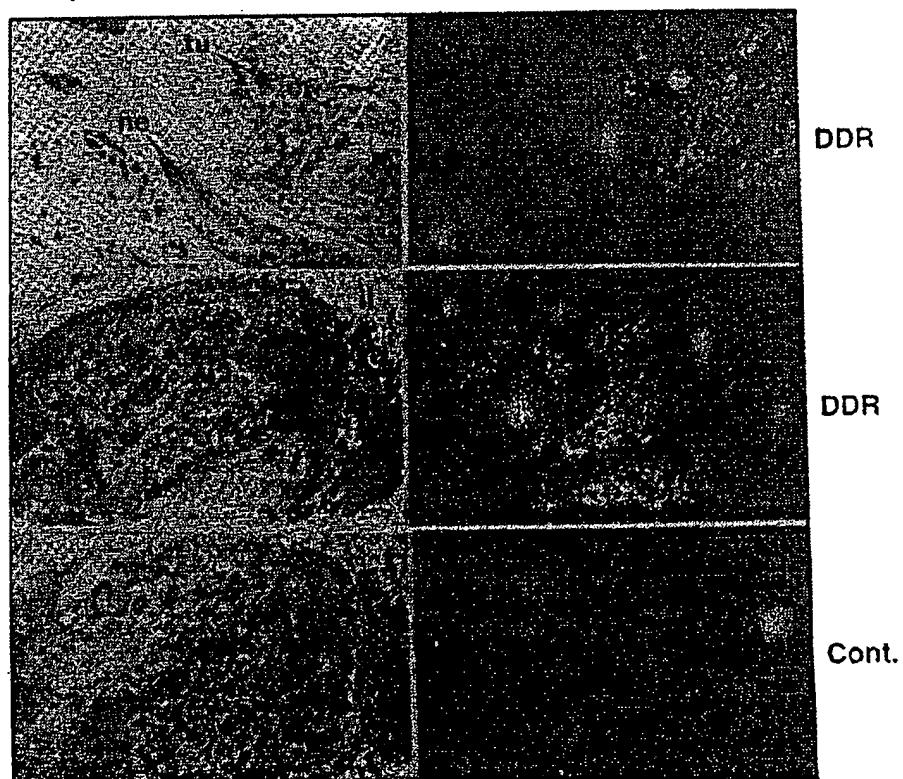


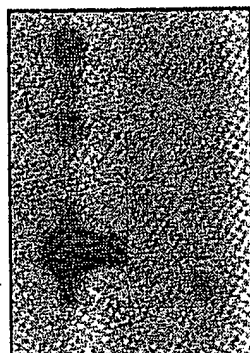
FIG. 6c



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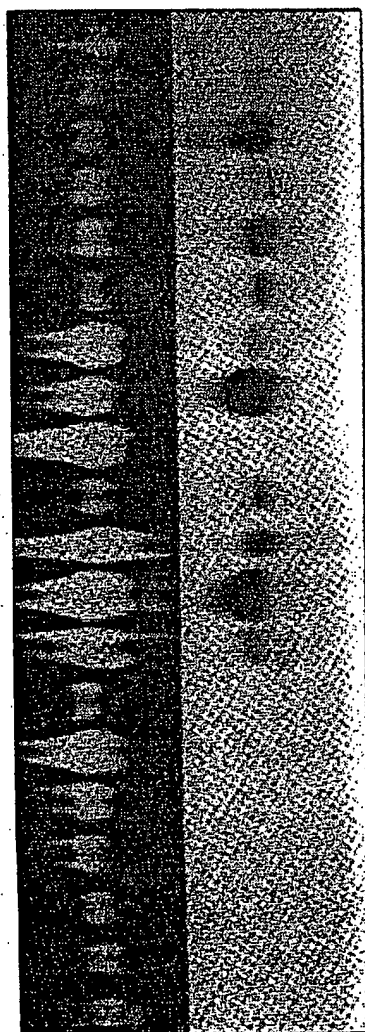
5/5

FIG. 7a



Heart
Brain
Placenta
Lung
Liver
Skeletal Muscle
Kidney
Pancreas

FIG. 7b



Oesophagus
Stomach
Gall Bladder
Ileum
Caecum
Colon
Tonsil
Thyroid
Spleen
Kidney Pelvis
Kidney Medulla
Kidney Cortex
Lung
Skin
Testis
Prostate
Myometrium
Endometrium
Breast
Bone Marrow

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 94/01480

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 G01N33/574 C12Q1/48 A61K31/55 A61K31/275 A61K31/35
 A61K39/395 A61K38/00 A61K31/70 G01N33/573 C12Q1/68
 C12N9/12 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N C12Q A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol.90, June 1993, WASHINGTON US pages 5677 - 5681 JEFFREY D. JOHNSON ET AL. 'A receptor tyrosine kinase found in breast carcinoma cells has an extracellular discoidin I-like domain' cited in the application see the whole document ---	1-25
Y	DRUGS OF THE FUTURE, vol.17, no.2, 1992 pages 119 - 131 TERRENCE R. BURKE, JR. 'Protein-tyrosine kinase inhibitors' cited in the application see the whole document --- -/--	1-25

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
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- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

25 October 1994

Date of mailing of the international search report

08.12.94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
 Fax (+ 31-70) 340-3016

Authorized officer

Döpfer, K-P

INTERNATIONAL SEARCH REPORT

Internat'l Application No

PCT/GB 94/01480

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF MEDICINAL CHEMISTRY, vol.34, 1991, WASHINGTON US pages 1896 - 1907 AVIV GAZIT ET AL. 'Tyrphostins. 2. Heterocyclic and alpha-substituted Benzylidenmalononitrile Tyrphostins as Potent Inhibitors of EGF Receptor and ErbB2/neu Tyrosine Kinases' cited in the application see the whole document ---	1-10, 19-21
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol.89, May 1992, WASHINGTON US pages 4285 - 4289 PAUL CARTER ET AL. 'Humanization of an anti-p185(HER2) antibody for human cancer therapy' cited in the application see the whole document ---	1-7, 11-13, 15, 19, 22-25
P,A	ONCOGENE, vol.8, no.12, December 1993, BASINGSTOKE GB pages 3433 - 3440 THOMAS KARN ET AL. 'Stzructure, expression and chromosomal mapping of TKT from man and mouse: a new subclass of receptor tyrosine kinases with a factor VIII-like domain' see the whole document ---	1-8, 11-19, 22-25
P,A	ONCOGENE, vol.9, no.1, January 1994, BASINGSTOKE GB pages 211 - 219 JOSE L. PEREZ ET AL. 'Identification and chromosomal mapping of a receptor tyrosine kinase with a putative phospholipid binding sequence in its ectodomain' see the whole document ---	1-8, 11-19, 22-25
P,A	ONCOGENE, vol.8, no.10, October 1993, BASINGSTOKE GB pages 2731 - 2739 MARIELBA ZERLIN ET AL. 'NEP: a novel receptor-like tyrosine kinase expressed in proliferating epithelia' cited in the application see the whole document ---	1-8, 11-19, 22-25
1 A	EP,A,0 504 914 (E.R. SQUIBB & SONS, INC.) 23 September 1992 see the whole document ---	1-8, 11-19, 22-25

-/--

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/01480

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,90 10234 (EURO-DIAGNOSTICS B.V.) 7 September 1990 see the whole document ---	1,4
A	SCIENCE, vol.241, no.4861, 1 July 1988, LANCASTER,PA, US pages 42 - 52 STEVEN H. HANKS, ANNE MARIE QUINN, TONY HUNTER 'The Protein Kinase Family: Conserved Features and Deduced Phylogeny of the Catalytic Domains' cited in the application ---	
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